

Adenomatous polyposis coli protein nucleates actin assembly and synergizes with the formin mDia1

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The tumor suppressor protein adenomatous polyposis coli (APC) regulates cell protrusion and cell migration, processes that require the coordinated regulation of actin and microtubule dynamics. APC localizes *in vivo* to microtubule plus ends and actin-rich cortical protrusions, and has well-documented direct effects on microtubule dynamics. However, its potential effects on actin dynamics have remained elusive. Here, we show that the C-terminal “basic” domain of APC (APC-B) potently nucleates the formation of actin filaments *in vitro*

and stimulates actin assembly in cells. Nucleation is achieved by a mechanism involving APC-B dimerization and recruitment of multiple actin monomers. Further, APC-B nucleation activity is synergistic with its *in vivo* binding partner, the formin mDia1. Together, APC-B and mDia1 overcome a dual cellular barrier to actin assembly imposed by profilin and capping protein. These observations define a new function for APC and support an emerging view of collaboration between distinct actin assembly-promoting factors with complementary activities.

Introduction

Dynamic reorganization of the actin cytoskeleton is indispensable for polarized cellular processes such as cell motility and asymmetric cell division. The *de novo* formation of actin filaments is a crucial step in these events. As such, cells require a variety of actin nucleators that are harnessed to the assembly of different actin structures (Chesarone and Goode, 2009). Further, the proper formation of actin structures underlying cell motility, cell polarization, and cytokinesis depends on closely coordinated interplay between the actin and microtubule cytoskeletons (Drubin and Nelson, 1996; Li and Gundersen, 2008).

Adenomatous polyposis coli (APC), a large 350-kD multidomain protein (Fig. 1 A), is a tumor suppressor linked to colorectal cancer and has an established role in regulating microtubule cytoskeleton organization and dynamics (McCartney and Näthke, 2008). In addition, APC has been suggested to influence actin cytoskeleton regulation, but the underlying mechanism has been unclear. Previous studies have shown that the C-terminal “Basic” region of APC (Fig. 1 A) binds to microtubules and stabilizes microtubules in cells together with its

binding partner EB1 (Munemitsu et al., 1994; Su et al., 1995; Kita et al., 2006). Loss of the C terminus of APC also causes defects in directional cell migration, a process that requires close coordination between the microtubule and actin cytoskeletons (Oshima et al., 1997). To date, the possible direct effects of APC on actin dynamics have not been well explored, and it has been postulated that the primary influence of APC on actin cytoskeleton remodeling occurs through indirect mechanisms, mediated by APC interactions with its binding partners, including its N-terminal associations with IQGAP and ASEF (Kawasaki et al., 2000; Watanabe et al., 2004) and its C-terminal associations with the formin mDia (Wen et al., 2004). However, recently it was shown that the C-terminal Basic domain of APC binds directly to F-actin (Moseley et al., 2007).

Here, we demonstrate that the APC-Basic domain induces actin assembly *in vivo* and potently nucleates actin polymerization *in vitro*, providing a direct mechanism for APC in regulating actin-based cell protrusion, motility, and polarity.

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Abbreviations used in this paper: APC, adenomatous polyposis coli; TIRF, total internal reflection fluorescence.

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Results and discussion

APC stimulates actin assembly in vivo and in vitro

To investigate effects of the C-terminal Basic (B) region of APC on cellular actin dynamics, we introduced a plasmid for expression of GFP-APC-B into serum-starved NIH3T3 cells by microinjection (Fig. 1 B). GFP-APC-B induced formation of bright-staining F-actin structures, and colocalized with them (Fig. 1 B). Quantification of the effects showed that GFP-APC-B caused a ~40% increase in total cellular F-actin content compared with cells injected with empty vector (Fig. 1 C). Further, when adjacent cells were microinjected, we observed a marked accumulation of F-actin at cell–cell contacts (Fig. 1 B, linescan). These observations suggested that APC-B might directly or indirectly induce actin filament assembly.

To investigate the mechanistic basis of these observations, we purified APC-B and a longer polypeptide (APC-C; Fig. 1 A) and tested their effects on actin assembly in vitro. Both polypeptides accelerated actin assembly in a concentration-dependent manner, with potent effects at low nanomolar concentrations (Fig. 1 D, APC-C; Fig. S2 A). Electron microscopy and total internal reflection fluorescence (TIRF) microscopy analysis of samples from early time points in the reactions showed that APC-B and APC-C induce formation of unbranched filaments (Fig. 1, E and F). APC-B did not sever filaments to promote disassembly (Fig. 1 G) or cap the growing barbed ends (Fig. 1 I) of preformed filaments to inhibit growth, demonstrating that the ability of APC-B to promote actin assembly does not stem from severing or capping. Further, APC-B–induced actin assembly was blocked by the barbed end-capping agent cytochalasin D (Fig. 1 H). Together, these data show that APC-B promotes robust de novo formation of actin filaments that grow primarily from their barbed ends.

Next, we directly compared the nucleation activities of APC-B and the FH1-FH2 domain-containing C-terminal half of mouse formin mDial1 (C-mDial1) at different actin concentrations (Fig. 2 A). Interestingly, APC-B was a more effective nucleator than C-mDial1 at low actin concentrations, which could be an important property in vivo under conditions where free actin monomers are limiting. Although formin-induced actin nucleation was partially suppressed in the presence of profilin, APC-B–induced nucleation was not (Fig. 2, B and C). Analysis of filament barbed end elongation rates in the presence of profilin by TIRF microscopy further revealed that, unlike formins, APC-B did not accelerate elongation (Fig. 2 D and Videos 1–3). These results are consistent with APC lacking recognizable profilin-binding motifs (Fig. S1), and highlight some of the major differences between APC-B and formins as actin assembly-promoting factors.

APC mechanism of actin nucleation

All nucleators identified to date use one of three mechanisms to promote de novo formation of actin filaments (Chesarone and Goode, 2009). Arp2/3 complex is proposed to structurally mimic the barbed end of an actin filament (Kelleher et al., 1995). Formins appear to stabilize spontaneously formed polymerization

intermediates, actin dimers and trimers (Pring et al., 2003). Spire, Cobl, Lmod, and JMY contain WH2 domains and recruit actin monomers to form pre-nucleation complexes that “seed” polymerization (Quinlan et al., 2005; Ahuja et al., 2007; Chereau et al., 2008; Zuchero et al., 2009). Our data above suggest that APC-B uses a mechanism distinct from Arp2/3 complex because APC-B promotes unbranched nucleation, and distinct from formins because APC-B does not protect barbed end growth from capping proteins or accelerate elongation. Therefore, we explored whether APC-B recruits actin monomers.

In native PAGE assays, G-actin alone migrated toward the anode, whereas APC-B alone migrated toward the cathode consistent with its high content of basic residues ($pI = 10.4$; Fig. 2 E). In the presence of APC-B, G-actin migration was reversed, comigrating with APC-B toward the cathode and increasing the density of the gel band (Fig. 2 E, bottom). Reactions contained 100 mM KCl, as well as latrunculin B, which blocks actin polymerization. These data suggest that APC-B physically associates with G-actin. Second, we tested APC-B–actin interactions by sedimentation velocity analysis in the presence of latrunculin B. Both APC-B and G-actin migration patterns shifted when mixed together compared with the migration patterns of each protein alone (Fig. S2, B and C), providing further support for their association. Third, we tested APC-B binding to actin monomers based on its ability to increase the fluorescence signal of pyrene-labeled G-actin (0.1 μM) under nonpolymerizing conditions including the presence of latrunculin B (Fig. 2 F). Binding was concentration dependent and saturated almost precisely at a 1:2 molar ratio of APC-B to G-actin (Fig. 2 F, dotted line) and with an apparent dissociation constant of 17.3 nM. Further addition of 5 μM profilin did not alter the effects of 50 nM APC-B on the fluorescence of 100 nM pyrene–G-actin (Fig. 2 G). This suggests that either APC-B and profilin have nonoverlapping binding sites on G-actin or APC-B very efficiently outcompetes profilin for G-actin binding, explaining why APC-B nucleation activity is not suppressed by the presence of profilin.

To further dissect the nucleation activity of APC, we generated a series of N- and C-terminally truncated APC-B polypeptides and compared their abilities to stimulate actin assembly and to bind G-actin (Fig. 3 A; Fig. S3, A–C). This analysis identified two sequences in APC-B that are critical for nucleation in vitro, ANS1 (residues 2326–2353) and ANS2 (residues 2526–2612). Moreover, constructs ΔN4 and ΔN5 , which both delete ANS1, drastically reduced APC-B–induced actin assembly in vivo (Fig. 3, B and C), suggesting that the ability of APC-B to promote actin assembly in vivo stems from its nucleation activity. Importantly, the truncations did not significantly alter the net charge of the APC-B polypeptides (listed in Fig. 3 A), indicating that nucleation effects are not due to nonspecific electrostatic attractions between APC-B and actin. The specificity of the nucleation effects was further demonstrated by the observation that ANS2 is not required for APC-B binding to microtubules (Fig. 3 A and Fig. S3 D).

We next tested the importance of ANS1 and ANS2 for APC-B binding to G-actin by comparing the effects of APC-B, ΔN4 , and ΔC2 in both the pyrene-actin fluorescence and native PAGE assays. In both assays, ANS1 was critical for APC-B

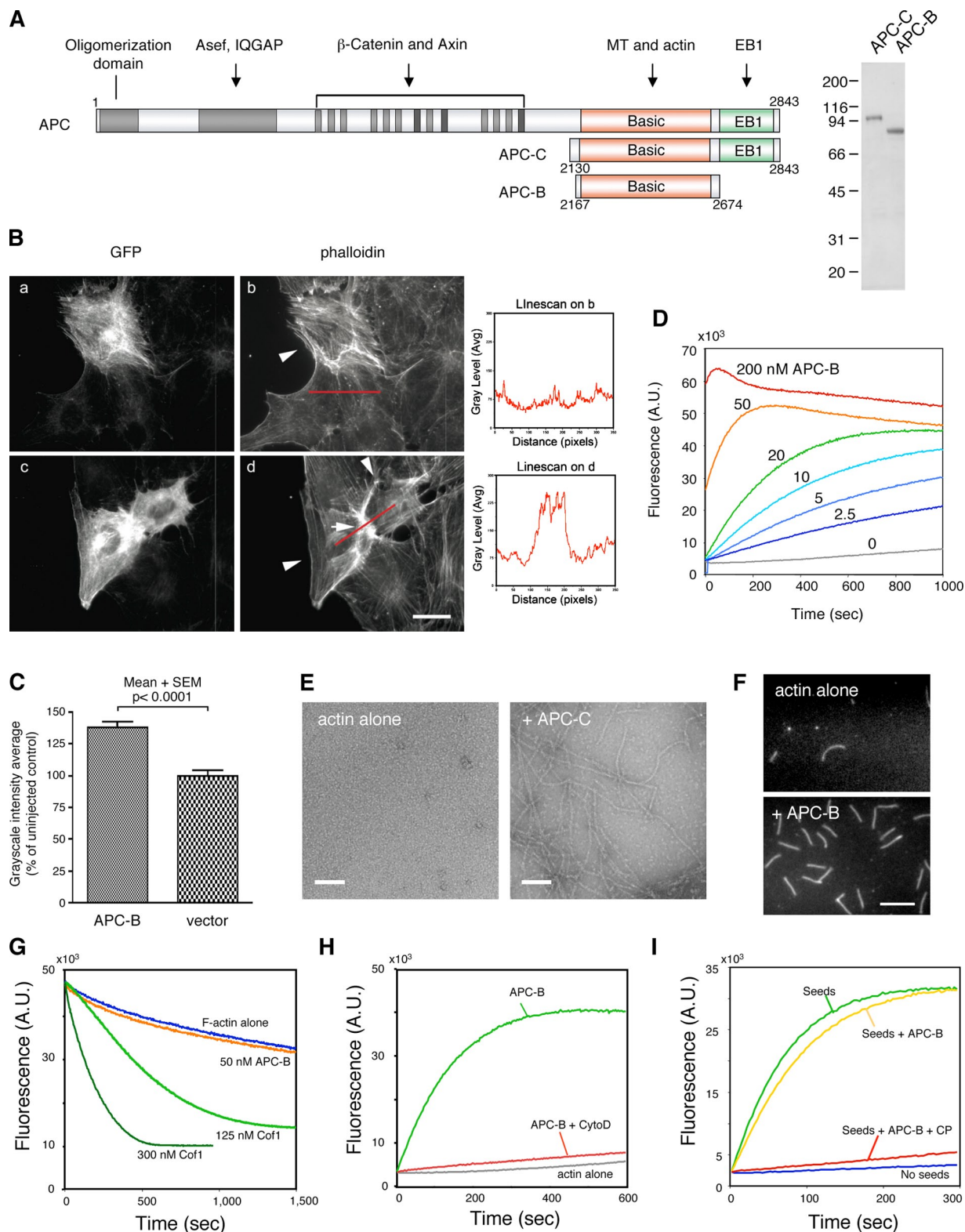


Figure 1. APC directly nucleates actin assembly. (A) Schematic of APC, and Coomassie-stained gel of purified APC polypeptides. (B) GFP fluorescence and rhodamine-phalloidin staining of serum-starved NIH3T3 cells. Arrowheads, cells microinjected with EGFP-APC-B plasmid. Arrow, F-actin accumulation at cell-cell junction. Bar, 10 μ m. Right panel shows linescan quantification of F-actin at cell-cell junctions in the absence (b, red line) or presence (d, red line) of GFP-APC-B. (C) Quantification of F-actin levels in cells ($n > 50$ cells). (D) Assembly of actin (2 μ M; 5% pyrene labeled) induced by 0–200 nM APC-B. (E) Electron micrographs of actin polymerized for 30 s in the presence or absence of 50 nM APC-C. Bar, 100 nm. (F) TIRF microscopy of actin (1 μ M; 30% labeled) assembled in the presence and absence of 2 nM APC-B, visualized 2–3 min after initiation of polymerization. Bar, 5 μ m. (G) Severing and depolymerization of 2 μ M preformed actin filaments was induced by Cof1 but not APC-B upon addition of 3 μ M Vitamin-D binding protein (a monomer sequestering factor) to induce depolymerization. (H) 100 nM cytochalasin D blocks the assembly of actin (2 μ M) stimulated by 20 nM APC-B. (I) APC-B does not protect filament barbed ends against 100 nM CapZ (CP) in seeded filament elongation assay.

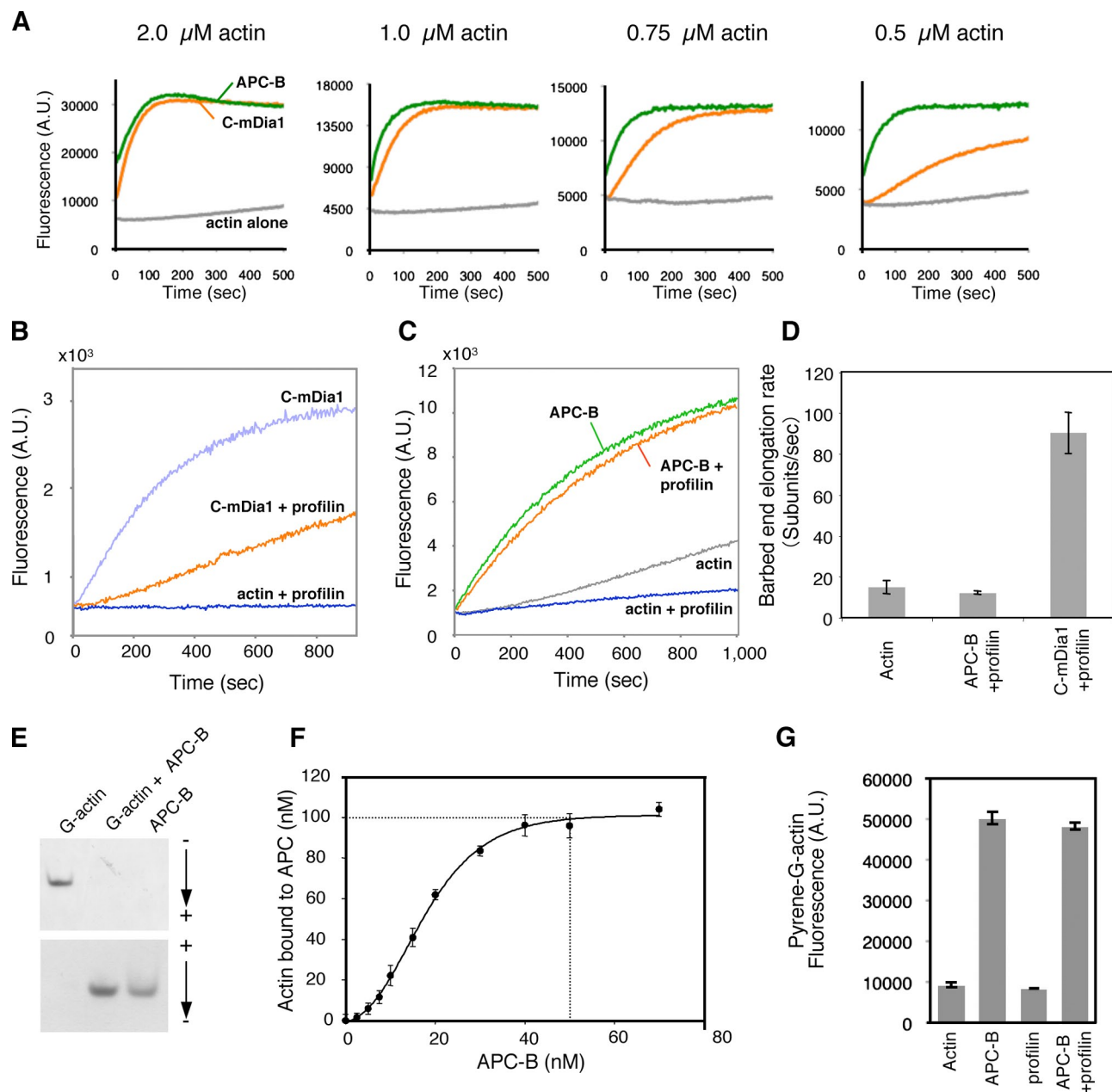


Figure 2. Properties of APC as an actin assembly-promoting factor. (A) Comparison of nucleation effects for 20 nM APC-B and 20 nM C-mDia1 at different concentrations of actin monomers (5% pyrene labeled). (B and C) Effects of 2 nM C-mDia1 or 20 nM APC-B on the assembly of actin (2 μM ; 5% pyrene labeled) in the presence or absence of 3 μM profilin. (D) Increase in barbed end elongation rate of individual actin filaments by C-mDia1 and profilin but not by APC-B. Rates of elongation were measured in real time by TIRF microscopy and averaged for >10 filaments. Error bars, standard deviation. (E) Native PAGE assay for APC-B binding to G-actin. Reactions were loaded on gels and run toward either the cathode (top) or anode (bottom), then gels were Coomassie stained. (F) Fluorescence-based assay for concentration-dependent binding of APC-B (0–75 nM) to G-actin (100 nM; 100% pyrene labeled). Dashed line indicates binding saturation at a 1:2 molar ratio of APC-B to actin. Error bars, standard deviation ($n = 3$). (G) 5 μM profilin does not affect the ability of 50 nM APC-B to increase the fluorescence of pyrene-G-actin (0.1 μM , 100% labeled). Error bars, standard deviation ($n = 3$).

binding to G-actin (Fig. S3, B, C, and E). On the other hand, deletion of ANS2 had no effect on APC-B binding to G-actin in pyrene fluorescence assays, yet disrupted formation of stable APC-B-actin complexes in native PAGE assays. Importantly, ΔC2 (which lacks ANS2) had the same 2:1 stoichiometry of APC-B-actin binding. These data suggest that ANS1 is crucial for actin binding, whereas ANS2 makes a distinct mechanistic contribution to formation of stable APC-B-actin complexes (see dimerization evidence below).

One major class of actin nucleators, which includes Spire, Cobl, Lmod, and JMY, contains tandem actin monomer-binding domains (typically WH2 domains) used to recruit up to four actin monomers into prenucleation complexes (Quinlan et al., 2005; Ahuja et al., 2007; Chereau et al., 2008; Zuchero et al., 2009). In Spire and Cobl, two WH2 domains are sufficient for weak nucleation, but all four are required for strong activity. Our stoichiometry data indicate that each APC-B molecule binds two actin monomers (Fig. 2 F). This prompted us to test

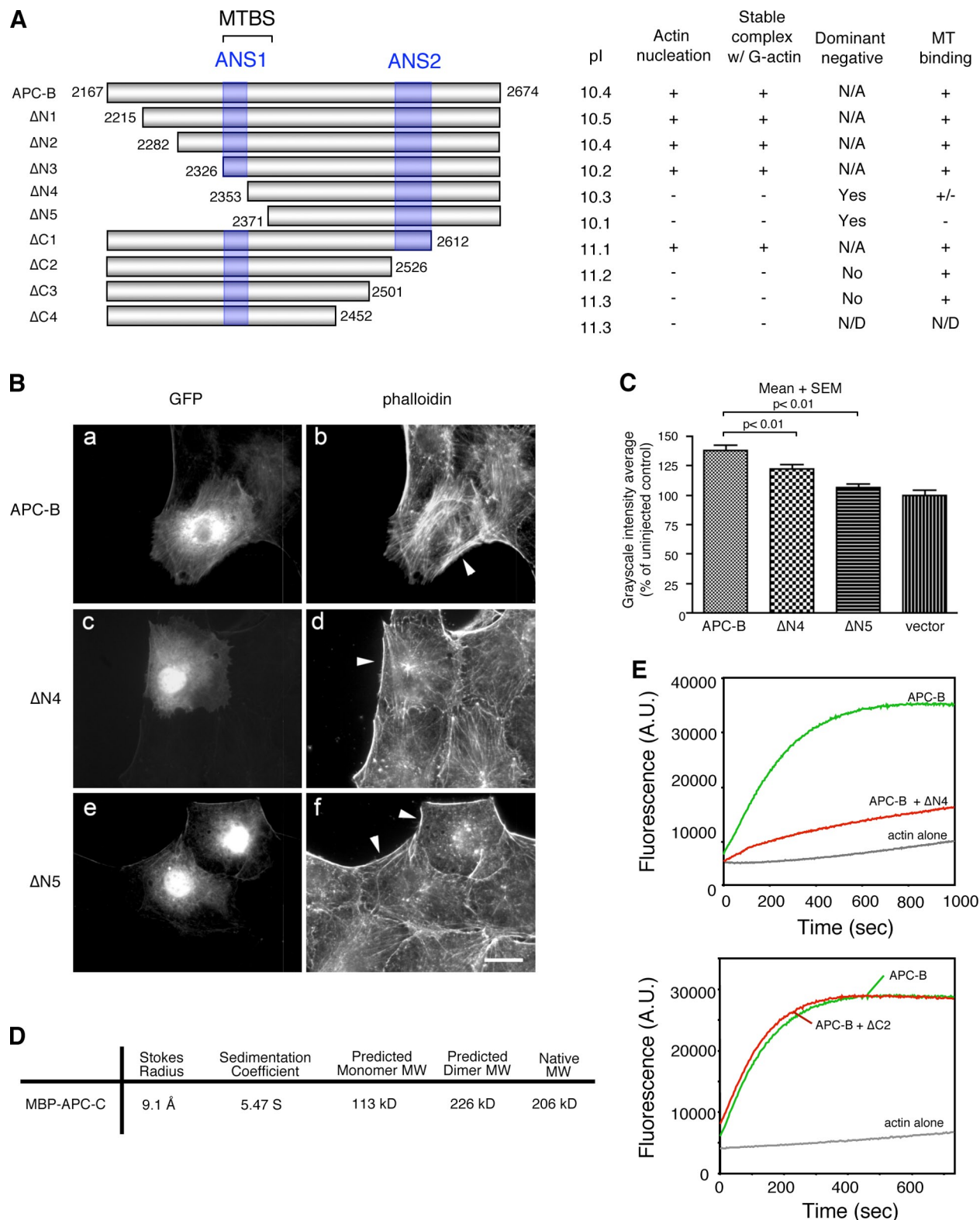


Figure 3. Dissection of APC-B actin nucleation activity and mechanism. (A) Schematic of APC-B truncated polypeptides analyzed. The actin nucleation sequences identified (ANS1 and ANS2) are shaded blue. MTBS, microtubule binding site; N/A, not applicable; N/D, not determined. (B) Deletion of ANS1 diminishes the actin nucleation activity of APC-B in cells. Serum-starved NIH3T3 cells microinjected with EGFP-APC-B, EGFP-APC-B-ΔN4, or EGFP-APC-B-ΔN5 plasmids (arrows) were fixed and imaged for GFP fluorescence and rhodamine-phalloidin staining. Bar, 10 μm. (C) Quantification of total cellular F-actin levels ($n > 50$ cells). (D) Dimerization of APC-C polypeptide. Stokes radius and sedimentation coefficient were determined for MBP-APC-C and used to calculate its native molecular weight (MW). Predicted MW of monomer and dimer for MBP-APC-C are listed for comparison. (E) Dominant-negative effects of truncated APC-B polypeptides on wild-type APC-B-induced actin assembly. Reactions contain actin (2 μM; 5% pyrene labeled) and 50 nM wild-type APC-B, with or without 50 nM APC-B-ΔN4 (top) or 50 nM APC-B-ΔC2 (bottom).

whether APC-B might dimerize to increase its actin-recruiting capacity. Previous studies have shown that the N terminus of APC dimerizes (Joslyn et al., 1993), but the properties of the C terminus have remained uncharacterized. Therefore, we determined the Stokes radii and sedimentation coefficients for APC-C and from these data calculated its native MW (Fig. 3 D), which suggested that APC-C forms a stable and extended dimer. A similar hydrodynamic analysis of truncation mutants showed that loss of ANS1 ($\Delta N4$) did not affect dimerization (multimerization state of 1.97), whereas loss of ANS2 ($\Delta C2$) weakened dimerization (multimerization state of 1.49). Further, $\Delta N4$ dominantly interfered with the actin nucleation activity of wild-type APC-B, whereas $\Delta C2$ did not (Fig. 3 E). The simplest interpretation of these results is that $\Delta N4$, which fails to bind actin, may interact with wild-type APC-B to form nucleation-incompetent heterodimers, whereas $\Delta C2$ is dimerization impaired and therefore does not interfere with wild-type APC-B activity. Given the 2:1 binding stoichiometry of actin to APC-B, we propose that each APC-B dimer can recruit up to four actin monomers in a prenucleation complex.

Synergy between APC-B and mDia1 in assembling actin filaments

We next explored the functional relationship of APC-B and mDia1 in actin assembly. As mentioned above, APC-B nucleates actin assembly efficiently in the presence of profilin, whereas C-mDia1 does not (Fig. 2, B and C). On the other hand, C-mDia1 protects growing barbed ends of filaments from capping proteins, whereas APC-B does not (Fig. 1 H). Given these complementary activities, and their reported physical interactions (Wen et al., 2004), we tested the combined effects of APC-B and C-mDia1 on actin assembly in the presence of profilin and capping protein, which presents a double barrier to actin assembly reflective of *in vivo* conditions (Fig. 4 A). APC-B alone and C-mDia1 alone showed only a weak ability to stimulate actin assembly under these conditions. However, combining the two proteins yielded strong synergistic effects. Together, APC-B and C-mDia1 produced a 3.6-fold higher actin assembly activity than the sum of their individual effects (Fig. 4 B). Consistent with these biochemical data, the ability of APC-B to induce actin assembly in NIH3T3 cells was greatly reduced after silencing of mDia1 expression by siRNA oligos but not control silencing of GAPDH (Fig. 4, C and D). Importantly, F-actin fluorescence intensities in cells did not differ significantly for mDia1 and GAPDH silencing alone (no APC injection). Together, these results show that APC-B and mDia1 can synergize to stimulate actin assembly both in a purified system and in living cells.

Based on these data, we propose the following model for APC-B/mDia1 synergy in assembling actin filaments (Fig. 4 E). APC-B dimers recruit up to four actin monomers to form a polymerization seed. This occurs efficiently in the presence of profilin. Next, mDia1 captures the barbed end of the nascent seed and moves processively with the growing end, accelerating elongation through FH1–profilin–actin interactions (Kovar and Pollard, 2004; Romero et al., 2004) while shielding growing ends from capping protein (Zigmond et al., 2003; Moseley et al., 2004).

Conclusions

Including APC, seven bona fide actin nucleators have now been identified, each with unique properties and mechanisms. The properties of APC-B as a nucleator are that it: (1) assembles unbranched filaments; (2) does not require a cofactor, whereas Arp2/3 complex does; (3) dimerizes and requires two functional halves (separate molecules) for nucleation; (4) fails to protect barbed ends of filaments from capping protein; (5) nucleates efficiently from profilin–actin; and (6) nucleates by recruiting up to four actin monomers, but does not contain recognizable WH2 sequences. Thus, APC appears to share at least some properties with nucleators of the Spir/Cobl/Lmod/JMY class, but also exhibits unique properties (most notably, it dimerizes and has no WH2 domains), suggesting that overall it has a novel mechanism of nucleation.

Because APC is well established as a microtubule regulator and binds to microtubules via its Basic region (Munemitsu et al., 1994), it was interesting to learn that the Basic region also nucleates actin assembly. Our truncation analysis showed that ANS2 is required for actin nucleation, and possibly dimerization, but not for microtubule binding (Fig. S3 D). Our data also indicate a close proximity and partial overlap of the microtubule-binding region of APC-B with ANS1; thus, steric hindrance could explain why APC-B interactions with actin and microtubules are mutually exclusive (Moseley et al., 2007). In addition, these observations raise the question of how APC interactions with actin and microtubules are differentially regulated *in vivo* and/or constrained to specific cellular compartments. This could involve post-translational modifications of APC and/or specific binding partners.

Our data also point to a strong synergy between APC-B and mDia1 in promoting actin assembly in the combined presence of profilin and capping protein *in vitro* and in cells (Fig. 4). This offers a functional explanation for the interactions between APC and mDia (Wen et al., 2004), although in the absence of more detailed information about their association, it is unclear whether synergy requires direct APC–mDia interactions. Nonetheless, this highlights how cells may combine two different actin assembly–promoting factors with unique strengths to bypass common inhibitors of nucleation and elongation. This synergy between APC-B and mDia1 could occur *in vivo* at actin-rich zones of membrane protrusion and cell–cell contact, where both APC and mDia1 have been localized (Watanabe et al., 1997; Rosin-Arbesfeld et al., 2001; Carramusa et al., 2007). Such a mechanism would offer increased spatiotemporal control over actin assembly *in vivo*, restricting new growth to locations where two separate factors had been successfully recruited and activated. This strategy may also extend to other pairs of actin assembly–promoting factors that have been shown to physically interact (e.g., Spire and formins, JMY, and Arp2/3 complex), and for which mounting genetic and biochemical evidence supports functional synergy (Bosch et al., 2007; Quinlan et al., 2007; Zuchero et al., 2009).

Finally, our results may have possible implications for how truncations in APC lead to colorectal tumor formation. Cancer-causing mutations in APC delete the entire C terminus, including the Basic domain. This stabilizes β -catenin, which is sufficient to induce tumor formation, but it has also been suggested

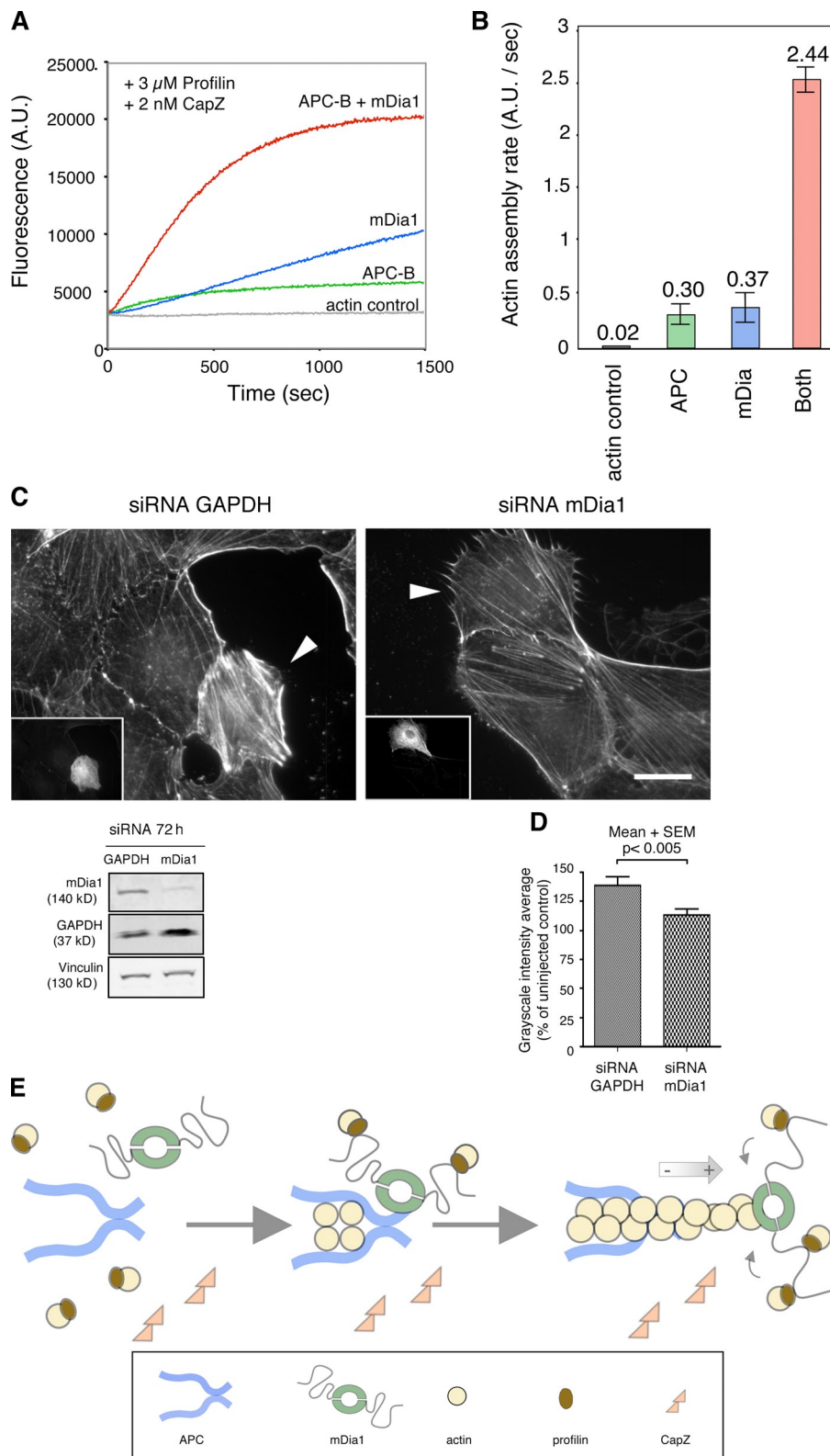


Figure 4. mDia1 and APC-B synergize to promote actin assembly in the combined presence of profilin and capping protein. (A) Reactions contain 2 μ M actin monomers (5% pyrene labeled), 3 μ M profilin, 2 nM CapZ, with or without 20 nM APC-B and/or 2 nM C-mDia1. (B) Quantification of elongation rates from the slopes of curves as in A. Rates are averages for reactions performed in triplicate in each of three separate experiments, where error bars represent standard error ($n = 3$). (C) Rhodamine-phalloidin staining of GAPDH or mDia1-depleted cells microinjected with EGFP-APC-B plasmid. Arrowheads point to injected cells. Insets show GFP signal for the same area. Bar, 10 μ m. Right panel is an immunoblot showing expression levels 72 h after siRNA transfection for GAPDH and mDia1, with vinculin as a loading control. (D) Quantification of F-actin levels induced by EGFP-APC-B in GAPDH or mDia1-depleted cells ($n > 40$). (E) Model for synergy between APC and mDia1 in promoting actin assembly, where APC efficiently seeds polymer formation by recruiting actin monomers from a pool of profilin-actin to form a pre-nucleation complex. The barbed end of the seed is captured by the FH2 domain of mDia1, which processively moves with the growing barbed end, protecting it from capping proteins while accelerating elongation through FH1–profilin–actin interactions.

that loss of the C-terminal activities of APC may contribute to tumor progression (Samowitz et al., 1999). Indeed, one of the earliest defects observed in the intestinal epithelium of APC truncation mutant mice lacking the C terminus is aberrant cell architecture and cell migration (Oshima et al., 1997; Sansom et al., 2004). Further, the C terminus of APC is sufficient to direct

localization to the actin-rich cell cortex and microtubule plus ends (Rosin-Arbesfeld et al., 2001). The C terminus of APC, which includes the Basic domain, is also indispensable for directional cell motility and normal protrusive activity in cultured cells (Kroboth et al., 2007). These observations have suggested that activities inherent to the C terminus of APC make fundamental

contributions to microtubule- and actin-based cell motility, leaving open the possibility that loss of such activities contributes to tumor progression. Future work that considers APC as a nucleator of actin assembly and a functionally synergizing binding partner of formins may help shed new light on how APC suppresses tumor formation.

Materials and methods

Plasmid construction

For expression of GFP-APC-B, GFP-APC-B- Δ N4, and GFP-APC-B- Δ N5 in NIH3T3 cells, coding sequences were PCR amplified from cDNA and subcloned into HindIII–BamHI sites of pEGFP-C3 (Takara Bio Inc.). For expression and purification of C-mDia1 (residues 549–1255) from yeast, the designated coding sequences were PCR amplified from cDNA and subcloned into pBG564 (URA3, 2 μ , *GAL1/10* promoter). APC-B and APC-C polypeptides were expressed in *Escherichia coli* using plasmids described previously (Moseley et al., 2007). To generate nested deletions in APC-B (Fig. 3 A), sequences were PCR amplified from a template plasmid and subcloned into EcoRI–NotI sites of the same vector. All plasmids were verified by DNA sequencing.

Cell culture and microinjection

NIH3T3 cells were grown in DME and 10% calf serum as described previously (Palazzo et al., 2001). For microinjection experiments, confluent monolayers of NIH3T3 cells were grown on acid-washed coverslips and serum starved for 48 h in DME plus 10 mM Hepes, pH 7.4. Cells were wounded 30 min before being microinjected in their nuclei as described previously (Palazzo et al., 2001) using 135 μ g/ml of plasmid DNA diluted in ddH₂O, and allowed to express for 3 h before fixation.

siRNA depletion

NIH3T3 cells were transfected with siRNA to silence GAPDH (5'-AAAGUUGUCAUGGAUGACCTT-3') or mDia1 (5'-AAGGUGAAGGAGGACCGCUUU-3') by using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's protocol. Protein levels were analyzed 72 h after transfection by immunoblotting with antibodies against GAPDH (rabbit 1:500; Santa Cruz Biotechnology, Inc.), mDia1 (mouse 1:500; BD), and vinculin (mouse 1:1,000; Sigma-Aldrich). For microinjection analysis, 24 h after transfection cells were serum starved for 48 h and injected with EGFP-APC-B cDNA, fixed, and processed for immunofluorescence as described below.

Epifluorescence microscopy and imaging analysis

Cells were fixed in 4% PFA in PBS for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Rhodamine-phalloidin (1:200; Invitrogen) was used to detect F-actin. Cells were observed on a microscope (Optiphot; Nikon) using a 60x PlanApo objective (1.4 NA) and filter cubes optimized for GFP and rhodamine fluorescence. Images were captured with a CCD camera (MicroMAX; KAF 1400 chip; Kodak) running MetaMorph software (MDS Analytical Technologies). To quantify cellular F-actin staining, one region was drawn around the perimeter of the injected cell and another around the perimeter of a noninjected control cell in the same frame. Gray intensity average values were measured by MetaMorph software and their ratio calculated after background subtraction using Excel (Microsoft). Statistical analysis of data was performed using Prism 4 (GraphPad Software, Inc.). All images shown in figures were processed and assembled using Photoshop (Adobe).

Protein purification

To circumvent issues of APC susceptibility to proteolytic degradation during its purification, we used separate affinity tags on either end (N-terminal GST, C-terminal 6His) and isolated APC-B and APC-C polypeptides from *E. coli* devoid of degraded products (Fig. 1 A). These proteins were purified sequentially on Ni-NTA (QIAGEN) and glutathione-agarose beads (GE Healthcare) as described previously (Moseley et al., 2007). Glutathione-eluted proteins were purified further by gel filtration on a Superose 6 column (GE Healthcare) equilibrated in 20 mM Tris-HCl, 600 mM KCl, 0.5 mM DTT, and 5% glycerol, pH 8.0. Peak fractions were pooled, exchanged into storage buffer (same as above except with 300 mM KCl), aliquoted, snap-frozen in liquid N₂, and stored at –80°C. MBP-APC-C polypeptide was purified on amylose resin (New England Biolabs, Inc.), then by gel filtration as above. C-mDia1 was purified as described previously (Moseley et al., 2006). Human profilin and chicken CapZ were expressed in *E. coli* and

purified as described previously (Soeno et al., 1998; Moseley et al., 2004). Rabbit skeletal muscle actin (RMA) was purified as described previously (Spudich and Watt, 1971) and gel filtered. For fluorometric assays, RMA was labeled with pyrenyl iodoacetamide (Cooper et al., 1983), and for TIRF analysis RMA was labeled on lysines with Alexa 532-succinimidyl-ester (Kellogg et al., 1988). Unlabeled tubulin was purified from bovine brain as described previously (Goode et al., 1999).

Actin assembly and disassembly assays

Gel-filtered Ca²⁺-ATP actin monomers (5% pyrene labeled) in G-buffer (3 mM Tris, 0.1 mM CaCl₂, 0.2 mM DTT, and 0.2 mM ATP, pH 7.5) were converted to Mg²⁺-ATP actin by adding 0.1 vol of 10 mM EGTA and 1 mM MgCl₂ and incubating for 2 min on ice. The actin was mixed with other proteins or control buffer, and polymerization was initiated by addition of 0.1 vol of 10x initiation mix (500 mM KCl, 20 mM MgCl₂, 10 mM EGTA, 2 mM ATP, and 200 mM Tris-HCl, pH 8.0). Pyrene fluorescence was monitored at excitation 365 nm and emission 407 nm in a fluorescence spectrophotometer (Photon Technology International). For seeded filament elongation assays (Fig. 1 I), preassembled F-actin (0.66 μ M in the final reaction) was sheared in the presence or absence of 20 nM APC-B and/or 100 nM CapZ, then mixed with 2 μ M G-actin (5% pyrene labeled) and monitored for polymerization. For actin disassembly assays (Fig. 1 G), preassembled F-actin (2 μ M final; 5% pyrene labeled) was mixed with Cof1 (125 or 300 nM), 50 nM APC-B, or control buffer, then Vitamin-D binding protein was added to initiate net disassembly.

Sedimentation velocity analyses and analytical gel filtration

Sedimentation coefficients (S value) of polypeptides were determined as described previously using sucrose gradient fractionation (Balcer et al., 2003). 10–50 μ g of purified polypeptide was loaded on a 12.6-ml sucrose gradient (3–30%) and centrifuged for 18 h at 4°C in a rotor (SW40 Ti; Beckman Coulter) at 30,000 rpm. 400 μ l fractions were harvested, and samples of each fraction were analyzed by immunoblotting. For gel filtration analysis, polypeptides were loaded on a Superose 6 column (AP Biotechnology). Elution peaks were monitored by absorbance at 280 nm and verified by immunoblotting column fractions. Size standards in both analyses were: aldolase (158 kD), albumin (67 kD), focal adhesion kinase FERM fragment (46 kD), and chymotrypsinogen A (25 kD). Native molecular weight was calculated from the sedimentation coefficient and Stokes radius as described previously (Schuyler and Pellman, 2002).

G-actin-binding analysis

For native PAGE assays, 3 μ l (0.5 μ g) of Ca-ATP-G-actin preincubated with latrunculin B in G-buffer was incubated for 10 min with or without 3 μ l APC-B (0.5 μ g; wild type or truncated) in storage buffer described above. Then 3 μ l of 3x loading buffer (75 mM Tris, 0.6 M glycine, 0.6 mM ATP, CaCl₂, 0.6 mM DTT, and 15% glycerol) was added before loading on native gel. Final reactions had 100 mM KCl and were fractionated on 7.5% native gels or 2.5% gels supplemented with 0.5% agarose as described previously (Moseley et al., 2004). For fluorescence-based assays, 100 nM Ca-ATP-G-actin (100% pyrene labeled) was preincubated for 5 min with 100 nM latrunculin B in G-buffer. Then 55 μ l of this mixture was added to 5 μ l of APC-B in storage buffer (0–75 nM final; see Fig. 2 F), and fluorescence levels were measured when reactions reached steady state.

Electron microscopy

G-actin (1.8 μ M) was mixed with APC-C (50 nM) or control buffer, and actin polymerization was initiated by addition of initiation mix. After 30 s, samples were adsorbed onto carbon-coated Cu grids for 50 s and stained with 2% (wt/vol) uranyl acetate. Images were collected on an electron microscope (Morgagni 268; FEI) operating at 80 kV.

TIRF microscopy

TIRF microscopy was performed as described previously with minor modifications (Kovar et al., 2006). Glass flow cells were incubated with 100 nM N-ethylmaleimide (NEM)-myosin for 2–5 min, washed extensively with 1% BSA, and equilibrated with TIRF buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 50 mM DTT, 0.2 mM ATP, 50 nM CaCl₂, 15 mM glucose, 20 μ g/ml catalase, 100 μ g/ml glucose oxidase, and 0.5% methylcellulose). 4 μ l of Mg-ATP actin monomers (30% Alexa 532 labeled) was mixed with 6 μ l of proteins (profilin, APC-B and/or C-mDia1; final concentrations in figure legend) or control buffer, and then added to 10 μ l of 2x TIRF buffer. Final reactions contained 1 μ M actin. Samples of reactions were transferred to a flow cell, and image acquisition was initiated within 2–5 min. Images were captured on a microscope (Eclipse GE2000-S; Nikon) equipped with an iXon EM-CCD camera (Andor Technology PLC)

and 100x PlanApo 1.40 NA objective with prism-based TIRF illumination from a 473-nm laser. Images were collected at 5-s intervals for 0.5-s exposure times. Data were processed using ImageJ (NIH, Bethesda, MD) and IDL version 6.3 (ITT Visual Information Solution).

Online supplemental material

Fig. S1 shows an alignment of APC-B sequences from multiple organisms. Fig. S2 shows a side-by-side activity comparison of APC-B and APC-C actin nucleation activities, and binding of APC-B to G-actin in 50 mM KCl. Fig. S3 shows the actin nucleation activities, G-actin-binding interactions, and microtubule-binding interactions of truncated APC-B polypeptides. Videos 1–3 show TIRF movies of actin filament assembly quantified in Fig. 2 D. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201001016/DC1>.

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Supplemental material

Okada et al., <http://www.jcb.org/cgi/content/full/jcb.201001016/DC1>

Human	2167	ILKPGEKSTLETKKIESESKGKGGKVKYKSLITGKVRNSEISSQMKQPLQANMPSISR	2226
Mouse	2167	ILKPGEKSTLEAKKIESENKGIKGGKVKYKSLITGKIRNSEISSQMKQPLPTNMPSISR	2226
Chicken	1555	ILKPGEKSTLESKKVESESKGKGGKRVYKSIITGKARSNVEVSSQIKQPQOTSVPSISR	1614
Xenopus	2166	ILKPAEKSALENKKTEEEPKGKGGKVKYKSLITGKSRSSDFSSHCKQSVQTNMPSISR	2225
		****.***:** ** *. * *****:*****:*** **.*:.*: ** . :.:*****	
Human	2227	GRTMIHIPGVRNSSSSTSPVSKKGPPLKTPASKSPSEGQTATTSRPGAKPSVKSELSPVA	2286
Mouse	2227	GRTMIHIPGLRNSSSSTSPVSKKGPPLKTPASKSPSEGGATTSPRGTKPAGKSELSPIT	2286
Chicken	1615	GRTMIHIPGVRNSSSSTSPVSKKGPFPKNTNSKSPSEGQSSASSPRGVKSSVKEPAPVT	1674
Xenopus	2226	GRTMIHIPGVRASPSTSPVSKKGPVFNVPKSGSNENPSSSSPKGKTP-LKSELVYGS	2284
		*****:* ** *.*****:*. * ..*. :.:**:*.*. **.* :	
Human	2287	RQTSQIG--GSSKAPSRSGSRDSTPSRPAQQPLSRPIQSPGRNSISPGRNGISPPNKLSQ	2344
Mouse	2287	RQTSQIS--GSNKGSSRSRSGRDSTPSRPTQQPLSRPMQSPGRNSISPGRNGISPPNKLSQ	2346
Chicken	1615	RQLSGLNQGGSSKGPSRSGSRDSTPSRPAQQPLSRPLQSPGRNSISPGRNGISPPNKLSQ	1734
Xenopus	2285	RPSSTPG--GSSKGNRSRSGSRDASSRSPQPLSRPLQSPGRNSISPGRNGISPPNKFSQ	2342
		* * . **.*. *****:.* ** *****:*****:*****:***	
Human	2345	LPRTSSPST ASTKSSGSGKMSYTSRGRQMSQQNLTKQTGLSKNASSI PRSESASKGLNQ	2404
Mouse	2345	LPRTSSPST ASTKSSGSGKMSYTSRGRQMSQQNLTKQASLSKNASSI PRSESASKGLNQ	2404
Chicken	1735	LPRTSSPST ASTKSSSGRMSYTSRGRQMSQQNLTKQTALTKNTSSI PRSESASKGLNQI	1794
Xenopus	2343	LPRTTSPST ASTKSSGSGRMSYTSRGRQMSQQNLTKQGLPKTHSSI PRSESASKGLNQ-	2401
		****:*****.**:*****:* ** *:*.*. *****.***	
Human	2405	NNGNANKKVELSRMSSTKSSGSESDRSERPVLRQSTFIKEAPSPTLRKLEESASFES	2464
Mouse	2405	SNNGSNKKVELSRMSSTKSSGSESDRSERPALVRQSTFIKEAPSPTLRKLEESASFES	2464
Chicken	1795	LGSASNKKTDLSRMSAKSSGSESDRSERPVLRQSTFIKEAPSPTLRKLEESASFES	1854
Xenopus	2402	NVNTGNSKKVELSRMSSTKSSGSESDRSERPALVRQSTFIKEAPSPTLRKLEESASFES	2461
		. :.*. :*****:*****.*****:*****:*****:*****	
Human	2465	LSPSSRPASPTRSQATPVLSPLPDMSLSTHSSVQAGGWRKLPNLSPTIEYNDGRPAK	2524
Mouse	2465	LSPSSRPDSPTRSQATPVLSPLPDMSLSTHSPVQAGGWRKLPNLSPTIEYNDGRPTK	2524
Chicken	1855	LSPS-RPDSPTRSQATPVLSPLPDMSLSTHSTAQTSRGRKLPNLSPSVEY-DGRPAK	1912
Xenopus	2462	LSSSRADSPRSQTQTPALSPSLPDMALSTH-SIQAGGWRKMPNLPAAEHGDSR--R	2518
		.* * . **.* ** *.***:*** : *.:***:***.* : * : *.* :	
Human	2525	RHDIARSHSESPSRLPINRSGTWKREHSKHSSSLPRVSTWRRTGSSSSILSASSESEKA	2584
Mouse	2525	RHDIARSHSESPSRLPINRAGTWKREHSKHSSSLPRVSTWRRTGSSSSILSASSESEKA	2584
Chicken	1913	RHDIARSHSESPSRLPINRSGTWKREHSKHSSSLPRVSTWRRTGSSSSILSASSESEKA	1972
Xenopus	2519	RHDIARSHSESPSRLPITRSGTWKREHSKHSSSLPRVSTWRRTGSSSSILSASSESEKA	2578
		****:*****.*.:*****:*****:*****:*****:*****:*****	
Human	2585	KSEDEK-HVNSISGTKQSKENQVSAKGTW RKIKENEFSPNTSQTVSSGA--TNGAESK	2641
Mouse	2585	KSEDER-HVSSMPAPRQMKENQVPTKGTW RKIKESDISPTGMASQSASSGA--ASGAESK	2641
Chicken	1973	KSEDEKQHGSSLSGQKQSKESQAPAKGTW RKIKENEIPQIMNDPQHPSSA--TSSSDSK	2030
Xenopus	2579	KSEDEKQVCSFPGPR--SECSAKGTW RKIKESEILETPNGSSSTIAE-SNCLESK	2635
		*****: : *.:. : . * . :*****:..: . : . : **	
Human	2642	TLIYQMAPAVSKTEDVWVRIEDCPINNPRSGRS	2674
Mouse	2642	PLIYQMAPPVSKTEDVWVRIEDCPINNPRSGRS	2674
Chicken	2031	TLIYQMAPAVSKTEDVWVRIEDCPINNPRSGRS	2063
Xenopus	2636	TLVYQMAPAVSKTEDVWVRIEDCPINNPRSGRS	2668

Figure S1. **Alignment of APC-B sequences from multiple organisms.** ANS1 and ANS2 sequences are shaded. GenBank accession numbers: human, NM_000038; mouse, NP_031488; chicken, XP_001233411; *Xenopus*, NP_001084352.

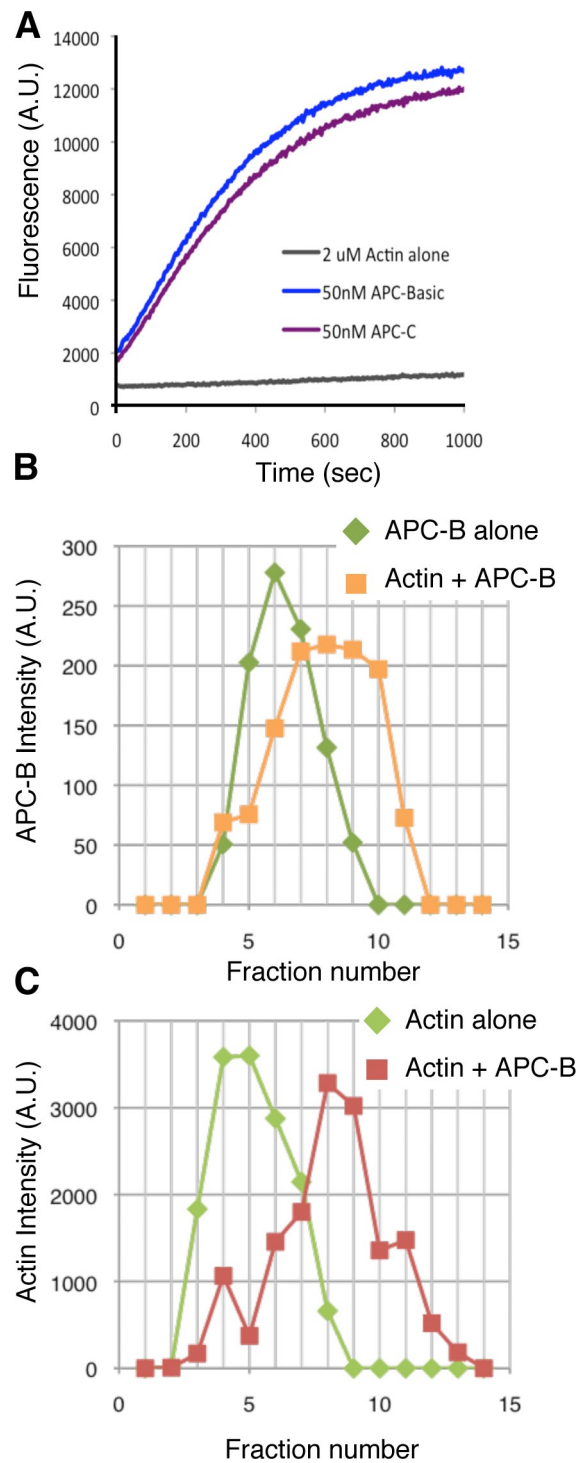


Figure S2. **Biochemical characterization of APC-B-actin interactions.** (A) Comparison of 50 nM APC-B and 50 nM APC-C on the assembly of 2 μ M actin (5% pyrene labeled). (B and C) Analysis of APC-B-actin interactions by sedimentation velocity analysis. 1 μ M G-actin preincubated with 2 μ M latrunculin B was mixed with or without 0.2 μ M APC-B in 20 mM Tris-HCl, 50 mM KCl, 0.1 mM ATP, 0.1 mM MgCl₂, and 0.5 mM DTT, incubated for 20 min, then fractionated on 3–30% sucrose gradients. Fractions were analyzed on gels by silver staining, and bands were quantified to compare migration patterns of APC-B (B) and actin (C).

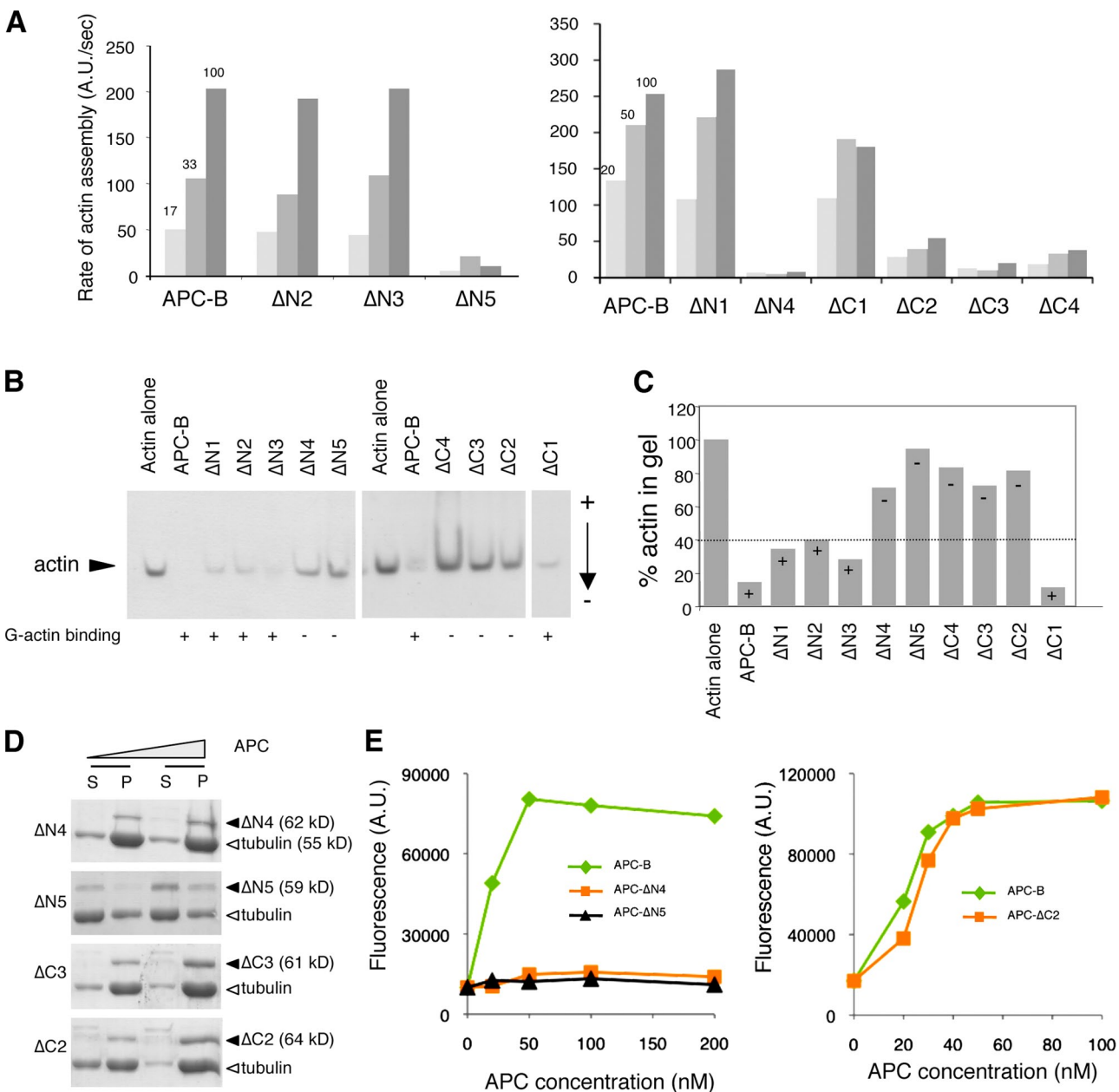
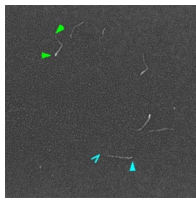
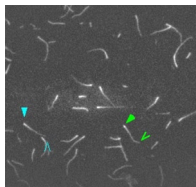


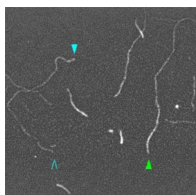
Figure S3. **Biochemical activities of truncated APC-B polypeptides.** (A) Actin nucleation activities of truncated APC-B polypeptides. Different concentrations (nM) of each construct, indicated above the bars in the graph, were compared with the same concentrations of wild-type APC-B for effects on assembly of 2 μ M actin (5% pyrene labeled). Rates of assembly were measured from slopes of curves. Data shown are representative of one to three experimental repeats. (B and C) G-actin binding by truncated APC-B polypeptides. Each construct was compared in native PAGE assays for its ability to shift the migration of G-actin. The disappearance of the actin band corresponds to binding by APC-B (see Fig. 2 E). Data shown are representative of two experimental repeats. (C) Intensities of G-actin bands in the gel were determined by densitometry and graphed as a percentage of actin-alone reactions. Assignments of APC-B-actin binding (+) and disrupted binding (-) for different APC-B constructs correspond to the values used in Fig. 3 A. (D) Microtubule binding activities of truncated APC-B polypeptides. Each construct was incubated for 20 min at room temperature with 2 μ M taxol-stabilized microtubules and centrifuged for 20 min at 50,000 rpm in a TLA-100 rotor. Supernatants (S) and pellets (P) were analyzed on Coomassie-stained gels. (E) Pyrene-G-actin (100 nM, 100% labeled) binding by selected APC-B truncates. Each construct was compared with wild-type APC-B for binding to G-actin by measuring fluorescence intensity increase of pyrene-G-actin as in Materials and methods.



Video 1. **This video corresponds to the rate of elongation for “actin” reactions in Fig. 2 D.** Time-lapse TIRF microscopy of 1 μM actin polymerization. Arrowheads point to representative actin filaments.



Video 2. **This video corresponds to the rate of elongation for “APC-B + profilin” reactions in Fig. 2 D.** Time-lapse TIRF microscopy of 1 μM polymerization in the presence 1.5 nM APC-B and 3 μM profilin. Arrowheads point to representative actin filaments.



Video 3. **This video corresponds to the rate of elongation for “C-mDia1 + profilin” reactions in Fig. 2 D.** Time-lapse TIRF microscopy of 1 μM actin polymerization in the presence 2 nM C-mDia1 and 3 μM profilin. Arrowheads point to representative actin filaments.

APC: More than a beta (catenin) blocker

Tumor suppressor could stop cancer through its effect on actin cytoskeleton.

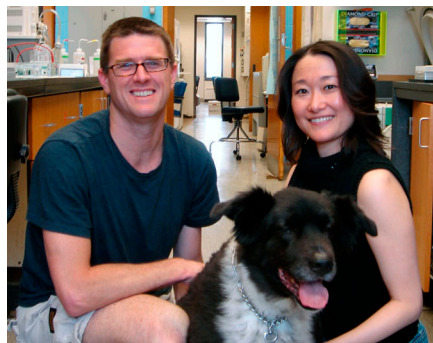
The adenomatous polyposis coli (APC) protein serves as the colon's guardian, keeping tumors at bay. Okada et al. (1) reveal a new function for the protein: helping to renovate the cytoskeleton by triggering actin assembly. The result suggests a second way that mutations in APC could lead to cancer.

A faulty APC gene occurs in more than 80% of colon cancers and is one of the early "gateway" mutations leading to abnormal growth. Researchers probing APC's anti-cancer powers have focused on how it curbs the activity of β -catenin, a key link in the Wnt pathway that manages cell division and differentiation (2). But APC also helps shape the cytoskeleton. The protein latches onto and stabilizes growing microtubule ends (3, 4) and connects to actin filaments (5). "There have been more and more clues in the literature that the loss of APC's cytoskeletal effects may contribute to cancer," says senior author Bruce Goode. Still unknown, however, was how APC alters the actin cytoskeleton.

To find out, Okada et al. experimented with a C-terminal fragment of APC that interacts with microtubules and actin. This pruned protein triggered actin polymerization *in vivo* and *in vitro*, the team showed.

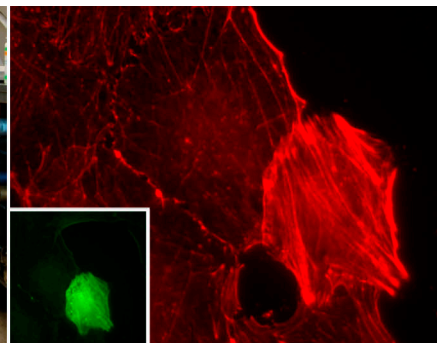
The researchers then compared APC's methods to those of other actin nucleators that spur filaments to grow. The Arp2/3 complex, for instance, almost tricks actin into polymerizing because it resembles the fast-growing barbed end of a filament. By contrast, researchers think that formins such as mDial

take a more passive approach, stepping in to stabilize short actin chains that form spontaneously. A third category, which includes Spire and Cobl, plays matchmaker, gathering actin monomers that unite into a polymer seed. Okada et al. found that APC dimerizes and then works like the Spire-containing group, corralling up to



A team led by Bruce Goode (left) and Kyoko Okada (shown here with lab dog Sasha) discovered that the tumor suppressor APC can trigger actin polymerization. This image of a layer of cells highlights the actin structures that formed in one cell after an injection of an APC fragment. The inset reveals that the GFP-tagged fragment latched onto actin.

FOCAL POINT



four actin monomers into a complex that seeds further elongation.

But that discovery raised another question. Cells deploy proteins that rein in actin extension. For example, profilin latches onto actin monomers and curbs spontaneous nucleation. And capping protein seals the barbed ends of actin filaments, preventing them from elongating and thus limiting their growth. APC can assemble actin filaments even if profilin is around. But how does it overcome capping protein?

The answer is that APC gets help, collaborating with formins that deter capping protein. Okada et al. found that when capping protein and profilin are present, APC or mDial alone is a weak nucleator. But combining the two boosts actin assembly nearly fourfold.

APC is the seventh actin nucleator that researchers have identified. "The cellular functions of actin are so pervasive," Goode says. "It's involved in dozens of critical processes, so it makes sense that cells have a large number of factors that promote actin assembly." So far, APC is the only nucleator with direct links to cancer. Goode says that it's plausible that APC mutations could foment

tumors not just through their effects on Wnt signaling, but also through their impact on the cytoskeleton. Cancer-causing mutations typically lop off the protein's actin-binding section.

Colon epithelial cells are born deep in crypts in the intestinal lining, and as they mature they differentiate, migrate into position, and give up their ability to divide. These choreographed changes involve precise rearrangements of the cytoskeleton and, if disrupted, could lead a cell down the path to cancer. By creating mice with a version of APC that can't nucleate actin, the researchers hope to nail down whether this mechanism contributes to tumor formation.

Because APC directly lengthens microtubules and actin, the protein could serve as an intermediary between the two cytoskeletons. So another question to investigate, the researchers say, is APC's role in situations where microtubules and actin remodel in concert, such as cell protrusion and migration.

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